Predictive role of cytokine gene polymorphisms for the development of femoral head osteonecrosis

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Abstract. *Introduction:* Osteonecrosis (ON) is a multifactorial disease that leads to hip destruction. Lately, much focus has been at femoral head preservation with nonsurgical methods. In this study we examined the polymorphisms of IL-1 α , IL-1R, IL-1RA, IL-4R α , IL-1 β , IL-12, γ IFN, TGF- β , TNF- α , IL-2, IL-4, IL-6 and IL-10 genes for evaluation of their contribution in ON.

Material and methods: DNA was extracted from 112 ON patients and 438 healthy donors. Analysis of the polymorphisms was completed using the PCR-SSP method. Statistical analysis was performed using the χ^2 test to compare the genotype and allelic frequency distribution.

Results: The CT and GA genotypes of the IL-1 α (-889) and TNF- α (-238) genes were found higher in the patients (51.8% and 10.8%, respectively) compared to the healthy donors (39.7% and 2.1%, respectively). In TGF- β codon 25, the G to C polymorphism in the homozygous state was found in 1.8% of the patients and the C allele frequency was 8.9%, whereas the G allele frequency was 91.1%. Also, at the IL-10 (-1082) gene the GG genotype was 16.2% in the controls whereas in the patients was 7.2%.

Conclusions: Based on the above, we showed that certain genotypes of the IL-1 α , TGF- β , IL-10 and TNF- α genes could be related in the pathogenesis of a complicated disease, such as osteonecrosis. The presence of one of the above mentioned polymorphisms or the simultaneous carriage of more than one may further increase the risk for osteonecrosis, especially in those at high risk, such as patients receiving corticosteroids.

Keywords: Osteonecrosis, avascular necrosis, cytokines, interleukins, osteoblasts, osteoclasts

1. Introduction

Osteonecrosis (ON) is a devastating disease usually leading to hip joint destruction in the third through fifth decades of life (average age, 36 years) [12,34,35,40]. The results of arthroplasty in these patients

were considered less than satisfactory, as failure rates up to 20% at a 5 year's follow up were initially reported [38]. However, recent studies with long term follow up (average of 17 years) report failure rates up to 17% [16]. ON is believed to be a multifactorial disease that is associated in some cases with both a genetic predilection as well as exposure to certain risk factors. These risk factors include corticosteroid use, alcohol intake, smoking and various chronic diseases (renal disease, hematological disease, inflammatory bowel disease, post-organ transplantation and hypertension) [1,7,11,14,19,20,25,26,41].

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The pathology of osteonecrosis involves ischemic events followed by death of bone and marrow elements. A process of repair is then initiated, but unless the lesion is small (less than 15% of the femoral head involved), this repair process is usually ineffective. The net result is weakening of subchondral bone with subsequent collapse of the articular surface [27]. This can lead to eventual collapse of the architectural bony structure of the femoral head, leading to joint pain and loss of function [3]. The vascular supply to the femoral head is affected by many endogenous and exogenous factors, such as thrombophilia, hypercortisonism and alcoholism. Common belief is that all of these factors run through diverse pathways to focal thrombosis and eventual osteonecrosis [15].

Nowadays, much focus has been on modalities aimed at femoral head preservation due to relatively poor results of hip arthroplasty treatment. Nonsurgical methods include pharmacologic agents and electrical stimulation. At least three of these factors are potential candidates as therapeutic modalities: cytokines, such as interleukins, tumour necrosis factors, and signalling molecules such as fibroblast growth factors, platelet derived growth factors, insulin-like growth factors, and transforming growth factor betas, bone morphogenetic proteins and angiogenic factors [1,35].

Cytokines are redundant secreted proteins with growth, differentiation and activation functions that regulate immune responses and immune response cell function as well as trafficking. The cytokine produced in response to an immune insult determines whether an immune response will be produced and whether this response will be humoral, cytotoxic, cell-mediated or allergic. A cascade of responses to cytokines can be seen in an immune response, as well as cytokines synergising for an optimal immune function. Cytokines could have a completely different function depending on the cellular source, target and of course the specific state of the immune response [4].

Several molecular pathways regulate the balance between osteoclasts and osteoblasts and determine the rate of bone remodeling. Certain cytokines such as IL-1, TNF- α and other proinflammatory cytokines stimulate osteoclasts differentiation and activation [36]. Also it is known that TNF- α acts on osteoblasts or bone marrow cells to synthesize and release cytokines whose role is directly associated with osteoclasts proliferation and maturation [9].

In this study we tried to evaluate the contribution of specific gene polymorphisms of several cytokines in osteonecrosis, a disease where osteoclasts-osteoblasts

balance is crucial for bone remodeling. Therefore, we examined samples from patients with osteonecrosis and normal samples for the polymorphisms within the genes of IL-1 α , IL-1R, IL-1RA, IL-4R α , IL-1 β , IL-12, γ IFN, TGF- β , TNF- α , IL-2, IL-4, IL-6 and IL-10.

2. Materials and methods

2.1. Study subjects

Five hundred and fifty subjects of Caucasian origin (Greece and Cyprus) were selected. Peripheral blood samples from 112 patients diagnosed with osteonecrosis at the stages II-IV (ARCO) and 438 of healthy donors between 20 and 50 years old were collected in the Department of Orthopaedic Surgery of a University Hospital. In relation to osteonecrosis etiology, 76 patients received steroids for some period of time and 5 had renal transplantation. Also, 4 had sickle cell anaemia, 2 were diagnosed with Systemic Lupus Erythematosus (SLE) and 5 other patients were alcoholic. In the remaining 20 patients no etiologic correlation was found and they were diagnosed as idiopathic cases. Healthy controls were derived from the same geographical area with no history of bone disease. The study was approved by the Scientific Committee of the University Hospital of Larissa (Greece) and is in accordance with the 1964 Declaration of Helsinki.

DNA from peripheral blood was extracted using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany). DNA quality and quantity was determined by electrophoresis in 1% agarose gel and absorbance spectrophotometry.

2.2. Cytokine genotyping

Polymorphisms were analyzed using the PCR-SSP method (Sequence Specific Primers) and the Protrans kit (Protrans HLA Cytokines 2, Roche Diagnostics, Mannheim, Germany). This specific kit uses PCR techniques with allele- and polymorphism- specific primers in the amplification reaction, recognizing 22 different polymorphisms in 13 cytokines. Each of the primer mixes contained as a control a primer pair that amplified either a part of the β -globin gene or a part of the C-reactive protein (CRP) gene. The β -globin control primers produce a 89-bp fragment, while the primer pairs amplifying the CRP gene produce a 440-bp amplicon. The allele and genotype frequencies of the following cytokine genes were determined: IL-1a (T/C -889),

Fig. 1. Electrophoresis of a sample for 13 different cytokines carried 22 polymorphisms. The allele specific amplification products for IL-1 α (lanes 1–2), IL-1 β (lanes 3–6), IL-1R (lanes 7–8), IL-1RA (lanes 9–10), IL-4R α (lanes 11–12), γ IFN (lanes 15–16), TGF- β (lanes 17–22) and TNF- α (lanes 23–26) are < 340 bp, for IL-12 (lanes 13–14) are > 800 bp and for IL-2 (lanes 27–30), IL-4 (lanes 31–38), IL-6 (lanes 39–42) and IL-10 (lanes 43–48) are > 300 bp. M: marker 100bp.

IL-1 β (C/T -511, T/C +3962), IL-1R (C/T pst11970), IL-1RA (T/C mspa111100), IL-4R α (G/A +1902), IL-12 (C/A -1188), interferon (IFN)- γ (A/T UTR 5644), transforming growth factor (TGF)- β (C/T codon 10, G/C codon 25), TNF- α (G/A -308, G/A -238), IL-2 (T/G -330, G/T +166), IL-4 (T/G -1098, T/C -590, T/C -33), IL-6 (G/C -174, G/A nt565), IL-10 (G/A -1082, C/T -819, C/A -592) (Fig. 1). All experiments were performed in triplicate.

2.3. Statistical analysis

Pearson χ^2 or Fisher's exact test was applied to analyze the comparison of frequencies of discrete variables between patients and controls. The odds ratio (OR) with 95% confidence interval (95% CI) were also calculated. All reported p values are two-tailed. Statistical significance was set at the $p < 0.05$ and analyses were conducted using SPSS statistical software (version 17.0).

3. Results

Allele and genotype frequencies are summarized in Tables 1 and 2. Genotype distributions of the examined genes were in Hardy-Weinberg equilibrium $p < 0.05$. The CT genotype in the IL-1 α at the -889 position was found in almost half of the patients (51.8%) with prevalence of T allele being 34.8%. In TGF- β codon 25, the G allele frequency was 91.1% in the patients

group, while the CC genotype was found only in 1.8% in the same group ($p < 0.05$). At position -238 of the TNF- α gene promoter, 10.8% of the patients had the GA genotype and only 2.1% of the control group. The allele frequency of the A allele was in both groups very low (patients: 5.4%, controls: 1.05%) (Table 1). Haplotypes were created for all the polymorphic sites of all examined genes; the GG/GG haplotype for the positions -308, -238 of the TNF α gene promoter was more frequent in both groups, whereas the GG/GA haplotype was more frequent in patients group ($p < 0.05$). At the -1082 position of the IL-10 gene the GG genotype was significantly higher in the control group ($p < 0.05$). Moreover, the GCC/GCC haplotype for -1082, -819 and -592 positions of IL-10 gene was more prevalent in the control group than in the patient group ($p < 0.05$) (Table 2).

4. Discussion

Osteonecrosis is a disease of unknown pathogenesis that usually progresses to hip joint destruction necessitating total hip arthroplasty. The pathology involves ischemic events followed by death of bone and marrow elements [27,28]. Considerable effort using in vitro and in vivo models has been directed toward understanding the effect of cytokines on osteoblasts and osteoclasts and their ultimate effect on bone resorption and formation, since these two types of cells are responsible for bone renewal. It is now apparent that the activities

Table 1
Genotype sequences of cytokine genes in ON patients and healthy group

	Genotype	Patients	Controls	P- value	OR (95% CI)
IL-1 α	CC	44 (39.3%)	211 (48.2%)	0.09	1.62 (1.07-2.47)
	CT	58 (51.8%)	174 (39.7%)	0.02	
	TT	10 (8.9%)	53 (12.1%)	0.34	
IL-1 β	CC	46 (41.1%)	197 (45%)	0.45	
	CT	49 (43.7%)	186 (42.4%)	0.80	
	TT	17 (15.2%)	55 (12.6%)	0.46	
IL-1 β	CC	50 (44.7%)	233 (53.2%)	0.10	
	TC	53 (47.3%)	183 (41.8%)	0.29	
	TT	9 (8%)	22 (5%)	0.22	
IL-1R	CC	54 (48.2%)	191 (43.6%)	0.38	
	CT	49 (43.8%)	192 (43.8%)	0.98	
	TT	9 (8%)	55 (12.6%)	0.18	
IL-1RA	TT	55 (49.1%)	211 (48.2%)	0.86	
	TC	49 (43.7%)	186 (42.5%)	0.80	
	CC	8 (7.2%)	41 (9.3%)	0.46	
IL-4Ra	AA	82 (73.2%)	340 (77.6%)	0.32	
	GA	27 (24.1%)	87 (19.9%)	0.32	
	GG	3 (2.7%)	11 (2.5%)	0.92	
IL-12	AA	69 (61.6%)	248 (56.6%)	0.34	
	CA	36 (32.1%)	162 (37%)	0.34	
	CC	7 (6.3%)	28 (6.4%)	0.95	
γ INF	AA	27 (24.1%)	116 (26.5%)	0.60	
	AT	58 (51.8%)	219 (50%)	0.73	
	TT	27 (24.1%)	103 (23.5%)	0.89	
TGF- β (codon 10)	CC	41 (36.6%)	139 (31.7%)	0.32	
	CT	44 (39.3%)	200 (45.7%)	0.22	
	TT	27 (24.1%)	99 (22.6%)	0.73	
TGF- β (codon 25)	GG	94 (84%)	352 (80.3%)	0.39	
	GC	16 (14.2%)	49 (11.2%)	0.36	
	CC	2 (1.8%)	37 (8.5%)	0.02	
TNF-a (-308)	GG	94 (84%)	369 (84.3%)	0.93	0.19 (0.04-0.83)
	GA	17 (15.2%)	58 (13.2%)	0.59	
	AA	1 (0.8%)	11 (2.5%)	0.31	
TNF-a (-238)	GG	100 (89.2%)	429 (97.9%)	0.04	0.17 (0.07-0.42)
	GA	12 (10.8%)	9 (2.1%)	0.00	
	AA	0 (0%)	0 (0%)	0.49	
IL-2 (-330)	TT	45 (40.2%)	168 (38.4)	0.72	
	TG	44 (39.3%)	202 (46.1%)	0.19	
	GG	23 (20.5%)	68 (15.5%)	0.20	
IL-2 (+166)	GG	62 (55.4%)	235 (53.6%)	0.74	
	GT	39 (34.8%)	165 (37.7%)	0.57	
	TT	11 (9.8%)	38 (8.7%)	0.70	
IL-4 (-1098)	TT	92 (82.1%)	360 (82.2%)	0.99	
	TG	18 (16.1%)	64 (14.6%)	0.69	
	GG	2 (1.8%)	14 (3.2%)	0.43	
IL-4 (-590)	TT	6 (5.4%)	17 (3.9%)	0.48	
	TC	22 (19.6%)	114 (26%)	0.16	
	CC	84 (75%)	307 (70.1%)	0.30	
IL-4 (-33)	TT	16 (14.3%)	98 (22.4%)	0.06	
	TC	11 (9.8%)	24 (5.5%)	0.09	
	CC	85 (75.9%)	316 (72.1%)	0.42	
IL-6 (-174)	GG	73 (65.2%)	248 (56.6%)	0.10	
	GC	35 (31.2%)	169 (38.6%)	0.15	
	CC	4 (3.6%)	21 (4.8%)	0.58	
IL-6 (nt565)	GG	74 (66.1%)	245 (56%)	0.05	
	GA	34 (30.3%)	153 (34.9%)	0.36	
	AA	4 (3.6%)	40 (9.1%)	0.06	
IL-10 (-1082)	GG	8 (7.2%)	71 (16.2%)	0.01	0.39 (0.18-0.85)
	GA	64 (57.1%)	220 (50.2%)	0.19	
	AA	40 (35.7%)	147 (33.6%)	0.66	
IL-10 (-819)	CC	64 (57.1%)	250 (57%)	0.99	
	CT	39 (34.8%)	167 (38.2%)	0.51	
	TT	9 (8.1%)	21 (4.8 %)	0.18	
IL-10 (-592)	CC	64 (57.1%)	250 (57%)	0.99	
	CA	39 (34.8%)	167 (38.2%)	0.51	
	AA	9 (8.1%)	21 (4.8 %)	0.18	

OR: Odds Ratio, CI: Confidence Interval.

Table 2
Haplotype frequencies in ON patients and controls

Cytokine	Position	Genotype	Patients	Controls	P-value	OR (95% CI)
TNFa	(-308-238/-308-238)	GG/GG	82 (73.2%)	360 (82.2%)	0.03	0.59 (0.36–0.96)
		GG/AG	17 (15.2%)	58 (13.2%)	0.59	
		GG/GA	12 (10.8%)	9 (2.1%)	0.00	5.72 (2.34–13.94)
		AG/AG	1 (0.8%)	11 (2.5%)	0.31	
IL-10	(-1082-819/-1082-819)	GC/AC	43 (38.3%)	110 (25.1%)	0.00	1.85 (1.19–2.87)
		GC/AT	21 (18.7%)	110 (25.1%)	0.15	
		GC/GC	8 (7.2%)	71 (16.2%)	0.01	0.39 (0.18–0.85)
		AC/AT	18 (16.1%)	57 (13.1%)	0.40	
		AC/AC	13 (11.6%)	69 (15.7%)	0.27	
		AT/AT	9 (8.1%)	21 (4.8%)	0.18	
IL-10	(-1082-592/-1082-592)	GC/AC	43 (38.3%)	110 (25.1%)	0.00	1.85 (1.19–2.87)
		GC/AA	21 (18.7%)	110 (25.1%)	0.15	
		GC/GC	8 (7.2%)	71 (16.2%)	0.01	0.39 (0.18–0.85)
		AC/AA	18 (16.1%)	57 (13.1%)	0.40	
		AC/AC	13 (11.6%)	69 (15.7%)	0.27	
		AA/AA	9 (8.1%)	21 (4.8%)	0.18	
IL-10	(-1082-819-592/-1082-819-592)	GCC/ACC	43 (38.3%)	110 (25.1%)	0.00	1.85 (1.19–2.87)
		GCC/ATA	21 (18.7%)	110 (25.1%)	0.15	
		ACC/ATA	18 (16.1%)	57 (13.1%)	0.40	0.39 (0.18–0.85)
		ACC/ACC	13 (11.6%)	69 (15.7%)	0.27	
		ATA/ATA	9 (8.1%)	21 (4.8%)	0.18	
		GCC/GCC	8 (7.2%)	71 (16.2%)	0.01	

OR: Odds Ratio, CI: Confidence Interval.

of these two bone cell types are interrelated. Osteoclastic activity, and therefore resorption, appears to be modulated by cytokines released by osteoblasts. Polymorphisms in cytokine genes may result in interindividual variation in transcriptional regulation, and thus in differential cytokine production. It has been widely hypothesized that genetic variants of cytokines could have phenotypic relevance and influence an individual's internal microenvironment [21,22,37,39].

The frequency of the studied cytokine polymorphisms in our subjects, which were Caucasians with a Greek and Cypriot origin, was similar to those referred by Costeas et al. in Greek Cypriot people, except for those frequencies concerning the codons 10 and 25 of TGF- β [8]. In more details, we showed that certain genotypes of the IL-1 α , TGF- β , IL-10 and TNF- α genes could be related in the pathogenesis of a complicated disease, such as osteonecrosis. This finding could be useful in the prognosis and treatment of people at high risk for osteonecrosis, such as corticosteroid users.

Interleukin (IL)-1 α is primarily a proinflammatory cytokine by its ability to stimulate the expression of genes associated with inflammation and immunity. Single nucleotide polymorphisms (SNPs) of the IL-1 α gene were reported to be associated with susceptibility to inflammatory diseases. More specifically, the SNP C/T (rs1800587) at -889nt in the 5'-flanking region of IL-1 α gene has been associated with susceptibility to

the inflammatory diseases, including periodontitis [18], juvenile idiopathic arthritis [23], psoriatic arthritis [33], Alzheimer's disease [32], preeclampsia [13], and systemic lupus erythematosus [30]; however, this SNP could predict the response of the treatment for rheumatoid arthritis [6] and the human immunodeficiency virus infection [31]. In our study, the CT genotype in the polymorphic site -889 was significantly higher in our patients group than the control group. Since IL-1 α is known to stimulate bone resorption via osteoclasts [17] and the T allele increases the protein level, thus it can be assumed that the CT heterozygotes carried higher protein levels resulting in an imbalance of osteoblasts/osteoclasts function.

TGF- β is a multifunctional cytokine that regulates proliferation and differentiation of a wide spectrum of cells both in vivo and in vitro. TGF- β influences cell growth, differentiation, apoptosis, cell migration, immune cell function and extracellular matrix production. It has been found that TGF- β production varies from person to person and is partly dependent on the different TGF- β gene polymorphisms. TGF- β pathway was described to modulate the development of several disease groups, including cancer, atherosclerosis, autoimmune and also fibrotic disease [2,5]. Our results indicate a higher percentage of homozygosity of the polymorphic C allele in the codon 25 of the TGF- β gene in the control group, while the G→C alteration in codon 25 (rs1800471) is associated with lower pro-

duction of TGF- β . Therefore, CC homozygotes from the normal subjects will have lower TGF- β production which could result in BMPs induction and be beneficial in bone regeneration, as it is known that TGF- β generally is considered to antagonize BMP-induced osteoblast differentiation [10].

Another anti-inflammatory cytokine, IL-10, is capable of inhibiting the synthesis of other pro-inflammatory cytokines such as IL-2, IL-3 and TNF α [24,42]. Our results showed that the haplotype GCC for the polymorphic sites -1082, -819 and -592 of IL-10 (rs 1800896, rs1800871 and rs1800872, respectively) gene was significantly associated with the control group. This haplotype is been responsible for a high capacity of IL-10 production which is in accordance with our findings. Also, the GC/GC genotypes for the IL-10 (-1082/-819) and IL-10 (-1082/-592) sites, appeared more frequent in the healthy donors. This specific genotype is associated with elevated levels of IL-10 and having in mind the inhibition role of IL-10 on other cytokines, we could hypothesize that especially the control group could benefit from this IL-10 excess, since this molecule has a restrictive role on other cytokines and their role.

A potent inflammatory cytokine, TNF- α , is released by macrophages in systemic inflammatory responses, regulating the inflammatory reaction and the immune response through promoting other cytokines expression. It is suggested that TNF- α acts on osteoblasts or bone marrow stromal cells to synthesize and release cytokines, such as IL-3, IL-6, IL-11 and IL-12 whose role is directly associated with the osteoclasts proliferation and maturation [9]. In fact, TNF- α acts as a skeletal catabolic agent that stimulates osteoclastogenesis while simultaneously inhibiting osteoblasts function [29]. TNF- α and RANKL are abundant in sites of inflammatory bone erosion and because these cytokines are potent osteoclastogenic factors and have overlapping signaling pathways, it is believed that under pro-inflammatory conditions RANKL and TNF- α might synergistically orchestrate enhanced osteoclastogenesis via cooperative mechanisms [43]. Our results demonstrate significant linkage of the genotype GA in TNF- α -238 polymorphic site in patients group. Moreover, the GG/GA haplotype at the -308 and -238 positions of TNF- α gene was higher in the patients group than the controls. The G $\rightarrow$ A alteration at the -238 (rs361525) site leads to an increased expression of the gene. Thus, it could be assumed that this augmentation in the patients group contributes in osteoclasts proliferation and loss of stability between osteoblasts and osteoclasts balance.

In conclusion, considerable effort using in vitro and in vivo models has been directed toward understanding

the effect of cytokines on osteoblasts and osteoclasts and their ultimate effect on bone resorption and formation. It is now apparent that the activities of these two bone cell types are interrelated and their balance determines the rate of bone remodeling. Polymorphisms in the genes of cytokines can affect this balance. The presence of one of the above mentioned polymorphisms or the simultaneous carriage of more than one may further increase the risk for osteonecrosis, especially in patients receiving corticosteroids. The application of cytokines in the prognosis and therapy of osteonecrosis shows promise, although clinical studies on efficacy and safety are needed.

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